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13. ABSTRACT ***(Maximum 200 Words)***

The objective of the project was to evaluate the role of TGF-β in prostate cancer progression. During the past funding period, we have made significant progress. We investigated if a non-immunogenic Dunning's rat prostate cancer cell line, MATLyLu, can become immunogenic by reducing the endogenous production of TGF-β1. An expression construct containing a DNA sequence in an antisense orientation to TGF-β1 (TGF-β1 antisense) was stably transfected into MATLyLu cells. Following transfection, cellular content of TGF-β1 reduced from 70 pg to 10 pg per $2x10^4$ cells and the rate of *in vitro* ^{3}H-thymidine incorporation increased 3-5 fold. After subcutaneous injection of tumor cells into syngeneic male hosts (Copenhagen rats), the tumor incidence was 100% (15/15) for the wild type MATLyLu cells and cells transfected with the control construct, but only 43% (9/21, $p \leq 0.05$) for cells transfected with TGF-β1 antisense. However, when cells were injected into immunodeficient hosts (athymic nude rats), the incidence of tumor development was 100% (10/10) for both the wild type MATLyLu cells and cells transfected with the control construct and 90% (9/10) for cells transfected with TGF-β1 antisense. These observations support the concept that MATLyLu cells are immunogenic, when the endogenous production of TGF-β1 is down regulated. The above work has been accepted for publication in the British Journal of Cancer.

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(5) INTRODUCTION

Although TGF-β is inhibitory to many cancer cells *in vitro* (Pietenpol et al, 1990; Laiho et al, 1990), most tumors that express large quantities of TGF-β exhibit an aggressive phenotype (Barrack, 1997). On the other hand, tumors that have a reduced production of TGF-β show an attenuated growth pattern *in vivo* (Fakhrai et al, 1996). This difference of *in vitro* and *in vivo* responses of tumor cells to TGF-β is well known. What remains unclear is the impact by the host immune system on *in vivo* tumor growth due to an overproduction of TGF-β. The present report described the role of TGF-β production in host immune surveillance program against tumor growth. Among the Dunning's rat prostate tumors, MATLyLu is the most aggressive line (Issacs et al, 1981; 1986). The MATLyLu system has been well characterized and is considered to be a good model for the late stage, aggressive form of human prostate cancer (Smolev et al, 1977). These cells are androgen independent and are highly invasive and metastatic. MATLyLu cells are known to be either non-immunogenic, or at best, weakly immunogenic (Shaw et al, 1987; Vieweg et al, 1994). They also produce a large amount of TGF-β1 (Steiner and Barrack, 1992; Barrack, 1997). Since TGF-β is a potent immunosuppresant (Letterio and Roberts, 1998), the lack of immunogenicity in these cells may be due, at least in part, to the large amount of endogenous production of TGF-β. MATLyLu cells are sensitive to TGF-β (Morton and Barrack, 1995). They demonstrate the typical paradoxical *in vitro* verses *in vivo* growth pattern in response to TGF-β1 (Barrack, 1997). Under culture conditions, TGF-β inhibits proliferation of MATLyLu cells. However, in animals, TGF-β1 enhances the tumorigenecity. This enhanced tumorigenicity may be the result of tumor-host interaction through a multitude of pathways. In the present study, we demonstrate that the immunosuppressive effect of TGF-β plays a major role in MATLyLu tumorigenicity. The present results allow us to conclude that MATLyLu cells can be immunogenic when the endogenous production of TGF-β has been suppressed.

(6) **BODY** (This section describes the research accomplishment associated with each Task outlined in the approved Statement of Work) A total of three tasks were proposed in the original application.

Task 1. To compare the differences in growth of MATLyLu cells under the *in vitro* and *in vivo* conditions when their ability to produce TGF-β1 has been reduced (months 1-6).

Work accomplished: We have completed most studies proposed in this task. Briefly, an expression construct containing a DNA sequence in an antisense orientation to TGF-β1 (TGF-β1 antisense) was stably transfected into MATLyLu cells. Following transfection, cellular content of TGF-β1 reduced significantly. Among 7 positive clones, clones 18 has the best performance. The endogenous production of TGF-β1 in clone 18 has reduced from 70 pg to 10 pg per $2x10^4$ cells and the rate of *in vitro* ^{3}H-thymidine incorporation increased 3-5 fold. Table 1 summarizes the level of TGF-β production and the rate of 3H-thymidine incorporation by the wild type MATLyLu cells, cells transfected with the control vector, and cells transfected with the TGF-β1 antisense vector.

Table 1. Levels of TGF-β1 in cell lysates and ^{3}H-thymidine incorporation rates of wild type MATLyLu cells, cells transfected with the control construct and cells transfected with the TGF-β1 antisense construct.

Cell Type	TGF-β1 (pg/$2x10^4$ cells)	^{3}H-Thymidine Incorporation (cpm/$1x10^4$ cells/well)
Wild Type MATLyLu Cells	70.3 ± 7.7	6,804 ± 434
Cells with Control Vector	67.0 ± 11.8	10,774 ± 700
Cells with Antisense Vector (Clone 18)	9.9 ± 1.2*	30,348 ± 2,866*

All values are expressed as mean ± standard error of the mean.
*The value is significantly different from the other values in the same column ($p < 0.05$).

Linear correlation was conducted to analyze the statistical association between the rate of ^{3}H-thymidine incorporation and the level of TGF-β1 production in 7 clones. The calculated correlation coefficient (r) was 0.769 and was statistically significant ($p < 0.05$). When these cells were injected subcutaneously into male Copenhagen rats (250 g. body weight), tumor incidence reduced to less than 50% of that for wild type MATLyLu (WT) cells and for cells transfected with an empty control vector (CV). Table 2 summarizes the tumor incidence in Copenhagen rats (syngeneic hosts) from the wild type MATLyLu cells, the control vector cells and the clone 18 cells.

Table 2. Tumor incidence of MATLyLu cells inoculated subcutaneously into syngeneic hosts.

	Trial I	Trial II	Trial III	Overall
Wild type MATLyLu cells	5/5(100%)	5/5(100%)	5/5(100%)	15/15(100%)
TGF-β1 antisense transfected cells	3/5(60%)	4/11(36%)*	2/5(40%)	9/21(43%)*
Control construct transfected cells	5/5(100%)	5/5(100%)	5/5(100%)	15/15(100%)

A total of $2x10^5$ cells were injected s.c.
*The value is significantly different ($p < 0.05$) from other values in the same group by the χ^2-square test (Steele and Torrie, 1960; Bender et al, 1982).

Work not completed: We shall conduct immunohistochemical staining for blood vessels in tumors derived from the wild type MATLyLu cells, cells transfected with the control vector and cells transfected with the antisense vector. This will be done in the next year.

Task 2: To evaluate the basic immunological parameters of different MATLyLu clones. Both *in vitro* and *in vivo* experiments will be conducted.

Work accomplished: We have performed irradiation of MATLyLu cells. The objective is to maintain the irradiated cells viable but unable to proliferate. We used the trypan exclusion assay to assess the cell viability and ^{3}H-thymidine incorporation assay to assess the ability of these cells to proliferate. A irradiation dose titration study was conducted. It was noted that MATLyLu cells required a large dose of radiation in order to render them to stop DNA synthesis yet remained viable. These irradiated cells will be used as the tumor vaccine to test their anti-tumor ability in an *in vivo* study.

Work to be completed: We shall conduct the *in vivo* study to test the efficacy of the tumor vaccine in its ability to reject the wild type MATLyLu cells, which will be used to challenge the Copenhagen male rats, according to our original proposal. At the end of this study, we shall assess the tumor incidence and the *in vitro* T-cell proliferation assay and cytotoxic assay will be conducted to support the *in vivo* study.

Task 3: To investigate the effect of TGF-β1 expression on non-immune responses in the host animals. Again, *in vitro* and *in vivo* experiments will be conducted.

Work accomplished: We have completed the task of xenograft growth of the genetically manipulated MATLyLu cells, which produced a reduced level of TGF-β1 in nude rats. Table 3 shows the results of that experiment.

Table 3. Tumor incidence of MATLyLu cells inoculated subcutaneously into immunodeficient hosts (nude rats).

	Trial I	Trial II	Overall
Wild type MATLyLu cells	5/5(100%)	5/5(100%)	10/10(100%)
TGF-β1 antisense transfected cells	5/5(100%)	4/5(100%)	9/10(90%)
Control construct transfected cells	5/5(100%)	5/5(100%)	10/10(100%)

A total of $2x10^5$ cells were injected s.c. Two trials were conducted for immunodeficient hosts.

It is interesting to note that, in contrast to results obtained in syngeneic hosts (see Table 2), the tumor incidence in nude rats was not significantly different among three types of MATLyLu cells.

Xenograft growth of tumor cells in athymic hosts offers an opportunity to assess the behavior of tumor development under immune compromised conditions. These animals are deficient in T cells but their natural killer (NK) cells remain functional. If tumors fail to develop in immune competent hosts but grow in immunodeficient hosts, it is likely that the observed difference in tumor incidence is at least due to a functional T-cell immune system. In the present study, this is the case, as the incidence has been significantly reduced in syngeneic hosts when compared with that in the nude rats. Therefore, a reduction in TGF-β1 production in the tumor cells results in an escape from the T cell mediated immunosuppression.

Work to be completed: We shall conduct studies to assess the production of extracellular matrix and to conduct the endothelial migration assay using these three types of MATLyLu cells in the next year.

(7) KEY RESEARCH ACCOMPLISHMENTS:

Although the proposed study has not yet completed, we have made significant insight into the role of TGF-β in MATLyLu tumor progression. Based on our observations made thus far, we are able to make the following conclusions.

- MATLyLu cells, when genetically manipulated to reduce their ability to production a large amount of TGF-β1, their ability to proliferate is enhanced under *in vitro* conditions but their ability to form tumors is retarded under *in vivo* conditions. This paradoxical observation was predicted by our original hypothesis. Now, we have experimental evidence to prove it.
- Our observations allowed us to conclude that the apparent *in vitro*-and-*in vivo* paradox in their proliferative pattern of MATLyLu cells is attributed to the fact that under *in vivo* conditions there is the host-tumor interaction, which is absent under *in vitro* conditions.
- Our observations further allowed us to conclude that the major force in this host-tumor interaction in the present system is the T-cell immunity, which is present in the syngeneic host (Copenhagen rats) but not in the immuno-deficient hosts (nude rats).
- MATLyLu cells has been considered as an aggressive high-grade rat prostate adenocarcinoma cells, which are known to be non-immunogenic. Many preclinical immuno-therapy protocols using MATLyLu cells have failed due to their characteristic non-immunogenic property. However, the present study has provided the opportunity to allow us to demonstrate that MATLyLu cells are highly immunogenic, if we reduce their ability to produce TGF-β1.

(8) REPORTABLE OUTCOMES

With this opportunity of funding from the Department of Defense, we were able to conduct the above-proposed experiments. The following publications are listed as our reportable outcomes.

- Lee C, Janulis L, Karpus WJ. (2000) Down regulation of TGF-β1 production restores immunogenicity in Dunning's rat prostate cancer cells (MATLyLu). Proceedings of the 91st Annual Meeting for the American Association for Cancer Research, April 1-5, San Francisco, California.
- Matthews E, Yang T, Janulis L, Goodwin S, Kundu SD, Karpus KJ, Lee C. (2000) Down regulation of TGF-β1 production restores immunogenicity in prostate cancer cells. British Journal of Cancer (Submitted).
- Both publications are enclosed as appendices.

(9) CONCLUSIONS

The concept that an elevated level of TGF-β production by the tumor cells can lead to a compromised host immune surveillance program in the host has been suggested before (Karpus and Swanborg, 1991; Fakhrai et al, 1996; Letterio and Roberts, 1998). However, the mere reduction of TGF-β production in these tumor cells can re-activate the host immune surveillance program has not been demonstrated before. Therefore, our present observations have allowed us to advance the concept of tumor immunology in that tumors that produce high levels of TGF-β are immuno-suppressive. The notion that most tumor cells produce elevated levels of TGF-β has been acknowledged. Our future studies should take advantage of this knowledge to develop a gene therapy program based on this concept.

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(11) APPENDICES

1. Lee C, Janulis L, Karpus WJ. (2000) Down regulation of TGF-β1 production restores immunogenicity in Dunning's rat prostate cancer cells (MATLyLu). Proceedings of the 91st Annual Meeting for the American Association for Cancer Research, April 1-5, San Francisco, California.
2. Matthews E, Yang T, Janulis L, Goodwin S, Kundu SD, Karpus KJ, Lee C. (2000) Down regulation of TGF-β1 production restores immunogenicity in prostate cancer cells. British Journal of Cancer (Submitted).

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The objective of this study is to determine if a non-immunogenic Dunning's rat prostate cancer cell line, MATLyLu, can become immunogenic by reducing the endogenous production of TGF-β1. An expression construct containing a DNA sequence in an antisense orientation to TGF-β1 (TGF-β1 antisense) was stably transfected into MATLyLu cells. Following transfection, cellular content of TGF-β1 reduced from 70 pg to 10 pg per $2x10^4$ cells and the rate of *in vitro* ^{3}H-thymidine incorporation increased 3-5 fold. After subcutaneous injection of tumor cells into syngeneic male hosts (Copenhagen rats), the tumor incidence was 100% (15/15) for the wild type MATLyLu cells and cells transfected with the control construct, but only 43% (9/21, $p \leq 0.05$) for cells transfected with TGF-β1 antisense. However, when cells were injected into immunodeficient hosts (athymic nude rats), the incidence of tumor development was 100% (10/10) for both the wild type MATLyLu cells and cells transfected with the control construct and 90% (9/10) for cells transfected with TGF-β1 antisense. These observations support the concept that MATLyLu cells are immunogenic, when the endogenous production of TGF-β1 is down regulated. (Supported by the Department of Defense, PC970410)

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Down Regulation Of TGF-β1 Production Restores Immunogenicity In Prostate Cancer Cells

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ABSTRACT

The objective of this study is to determine if a non-immunogenic Dunning's rat prostate cancer cell line, MATLyLu, can become immunogenic by reducing the endogenous production of TGF-β1. An expression construct containing a DNA sequence in an antisense orientation to TGF-β1 (TGF-β1 antisense) was stably transfected into MATLyLu cells. Following transfection, cellular content of TGF-β1 reduced from 70 pg to 10 pg per $2x10^4$ cells and the rate of *in vitro* ^{3}H-thymidine incorporation increased 3-5 fold. After subcutaneous injection of tumor cells into syngeneic male hosts (Copenhagen rats), the tumor incidence was 100% (15/15) for the wild type MATLyLu cells and cells transfected with the control construct, but only 43% (9/21, $p \leq 0.05$) for cells transfected with TGF-β1 antisense. However, when cells were injected into immunodeficient hosts (athymic nude rats), the incidence of tumor development was 100% (10/10) for both the wild type MATLyLu cells and cells transfected with the control construct and 90% (9/10) for cells transfected with TGF-β1 antisense. These observations support the concept that MATLyLu cells are immunogenic, when the endogenous production of TGF-β1 is down regulated.

Key words: TGF-β expression, rat prostate cancer, immunogenicity, tumor incidence, host-tumor interaction.

INTRODUCTION

Although TGF-β is inhibitory to many cancer cells *in vitro* (Pietenpol et al, 1990; Laiho et al, 1990), most tumors that express large quantities of TGF-β exhibit an aggressive phenotype (Barrack, 1997). On the other hand, tumors that have a reduced production of TGF-β show an attenuated growth pattern *in vivo* (Fakhrai et al, 1996). This difference of *in vitro* and *in vivo* responses of tumor cells to TGF-β is well known. What remains unclear is the impact by the host immune system on in vivo tumor growth due to an overproduction of TGF-β. The present report described the role of TGF-β production in host immune surveillance program against tumor growth.

Among the Dunning's rat prostate tumors, MATLyLu is the most aggressive line (Issacs et al, 1981; 1986). The MATLyLu system has been well characterized and is considered to be a good model for the late stage, aggressive form of human prostate cancer (Smolev et al, 1977). These cells are androgen independent and are highly invasive and metastatic. MATLyLu cells are known to be either non-immunogenic, or at best, weakly immunogenic (Shaw et al, 1987; Vieweg et al, 1994). They also produce a large amount of TGF-β1 (Steiner and Barrack, 1992; Barrack, 1997). Since TGF-β is a potent immunosuppresant (Letterio and Roberts, 1998), the lack of immunogenicity in these cells may be due, at least in part, to the large amount of endogenous production of TGF-β. MATLyLu cells are sensitive to TGF-β (Morton and Barrack, 1995). They demonstrate the typical paradoxical *in vitro* verses *in vivo* growth pattern in response to TGF-β1 (Barrack, 1997). Under culture conditions, TGF-β inhibits proliferation of MATLyLu cells. However, in animals, TGF-β1 enhances the tumorigenecity. This enhanced tumorigenicity may be the result of tumor-host interaction through a multitude of pathways.

In the present study, we demonstrate that the immunosuppressive effect of TGF-β plays a major role in MATLyLu tumorigenicity. The present results allow us to conclude that MATLyLu cells can be immunogenic when the endogenous production of TGF-β has been suppressed.

MATERIALS AND METHODS

MATLyLu cells and culture conditions: MATLyLu cells were kindly provided by Dr. John Isaacs of Johns Hopkins University at passage 53. Cells were routinely maintained in RPMI 1640 medium (Gibco Life Technologies, Gaithersburg, MD) with 10% FBS (Summit, Ft. Collins, CO), Penicillin (100 units/ml), Streptomycin (100 μg/ml) (Gibco), and 250 nM dexamethasone. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells under selective pressure were cultured as above with the addition of G418-sulphate (1,000 μg/ml). Transfection of expression constructs to MATLyLu cells was performed at passage 71 and cells were used for the present study between passages 80 to 90.

Construction of the TGF-β1 antisense expression vector: The pTARGET plasmid (Promega, Madison, WI) is a mammalian expression vector containing the human cytomegalovirus immediate-early promoter region to allow constitutive expression of the cloned vector in host cells. This plasmid also contains a neomycin resistance gene, which was used for selection by G418-sulphate. The vector consisted of base pairs (bp) 433-1461 of the rat TGF-β1 cDNA in the reverse orientation and was inserted into the multiple cloning site of the pTARGET plasmid. Resulting constructs were digested with restriction enzymes and sequenced to confirm correct orientation, and designated as the TGF-β1 antisense construct. A construct with the empty vector (pTARGET plasmid alone) was prepared in the same manner, and designated as the control construct

Transfection and Cloning: MATLyLu cells were transfected with the TGF-β1 antisense and control constructs with the Lipofectamine™ transfection system (Gibco) according to the manufacturer's recommended procedure. Briefly, cells were suspended in Opti-mem transfection media and were treated with a mixture of Lipofectamine™ and the above constructs (TGF-β1 antisense or control) for 18 hours. Following transfection, cells were cultured with the maintenance medium and selected with G418-sulphate. Three days later, these cultures were transfected again and this procedure was repeated for a

total of five times. Cloning was performed by limiting dilution into 96-well plates (Costar, Cambridge, MA). Seven days after the initial plating, cells in a single colony were harvested and transferred into 24-well plates. At confluency, these cells were subsequently transferred into 25 cm^2 flasks and further expanded. Prior to injection of these cells into animals, the following tests were conducted.

Verification of cloned MATLyLu cells by PCR: Confluent cultures were trypsinized and DNA was extracted via the Qiagen DNA preparation kit (Qiagen Inc, Chatsworth, CA). DNA contents were measured by UV absorbence. An aliquot of 1.0 μg of genomic DNA was introduced into each PCR reaction vessel along with primers specific for a sequence within the vector. DNA sequences for the respective primers are listed below.

5' primer-5'-GCACC AAAAT CAACG GGACT-3' (bp 619-638)

3' primer-5'-GAGAG AAAGG CAAAG TGGAT GTC-3' (bp 995-1017).

An aliquot of 10 μl reaction buffer, containing 0.5 μg of Taq polymerase (ISC Bioexpress, Kaysville, UT), 25 mM of dNTPS, 3 mM of $MgCl_2$, was added into each PCR reaction vessel and incubated for 35 cycles in a thermocycler (MJ Research, Watertown, MA). Each cycle consisted of 95°C for 2 minutes, 55°C for 1 minute, and 72°C for 1.5 minutes. The PCR products were subjected to electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, and were visualized under a UV lamp. The expected size (399 bp) of the PCR product was determined according to the DNA size reference, which was placed in the first and last lanes in the agarose gel for electrophoresis. Portions of the PCR products were also subjected to restriction digest and, then, to agarose electrophoresis to confirm the expected sequence.

***In vitro* ^{3}H-thymidine incorporation:** ^{3}H-thymidine incorporation was carried out to assess proliferative potential of each clones. Cells were seeded at 10,000 cells/well in 96-well plates and cultured in serum-free media. Twenty-four hours later, 1.0 μCi of ^{3}H-Thymidine (specific activity, 20 to 30 Ci/mmol; Amersham, Piscataway, NJ) was added to each well. Cells were incubated for an additional 2 hours and were subjected to two freeze-thaw cycles to detach cells from the wells. Suspended cells were

transferred to a glass filter (Packard Instrument Company, Meriden, CT) and were lysed with distilled water. Plates were allowed to dry for 2 hours and an aliquot of 50 µl scintillation fluid was added. The amount of radioactivity was measured in a Scintillation Top Count Plate Reader (Packard Instrument Company). Results were expressed as counts per minute per well.

Preparation of tumor and cell lysates for TGF-β1 determination: Levels of TGF-β1 in cell lysates and tumor tissues were determined by enzyme linked immunosorbant assay (ELISA). Tumor tissues, weighing between 200 and 600 mg, were frozen with liquid nitrogen and ground to a powder. The solubilizing solution was added to each sample at a rate of 3 ml (phosphate buffer, 100 µg leupeptin) per 100 mg of tissue. Cell lysates were prepared by addition of 3 ml of the solubilizing solution to cell pellets (10×10^6 cells). These samples were subjected to constant shaking at room temperature for 4 hours and centrifuged for 15 minutes at 1,500 x *g*. The clear supernatants were used for TGF-β1 determination. Briefly, an aliquot of 100 µl of the supernatant was activated with 100 µl 2.5 N HCl and 10 M Urea for 10 minutes. The activation step was necessary, as the amount of TGF-β1 in untreated samples was always negligible. Samples were neutralized with 100 µl of 2.7 M NaOH and 1.0 M HEPES prior to assay for TGF-β1. Samples were diluted 1:30 in calibrator diluent and subjected to ELISA for TGF-β1 (R&D, Minneapolis, MN) according to the manufacturer's suggested procedure. Results were reported as pg TGF-β1 per 50 µg of tumor tissue or per $2x10^4$ cells.

Experimental Animals: Copenhagen male rats (syngeneic hosts), Lewis male rats (allogeneic hosts), and athymic nude rats (immuno-deficient hosts) were purchased from Harlan Industry (Indianapolis, IN) at 60 days of age. Animals were kept in a temperature controlled room (23±2°C) with tap water and normal rat chow provided ad lib. Experiments started at least one week after animals arrived at the facility. They were anesthetized under methoxyflurane vapor (Schering-Plough, Union, NJ). MATLyLu cells (2×10^5 cells for syngeneic hosts and immunodeficient hosts, $1x\ 10^6$ cells for allogeneic hosts) were suspended in 0.2 ml of serum-free culture medium and injected subcutaneously with a 25-

gauge needle into the left flank near the hind leg, while the animals were under anesthesia. All procedures were approved by the Institutional Animal Care and Use Committee

Tumor Measurement and Tumor Histology: Seven days following injection, rats were palpated twice weekly in order to monitor tumor development and tumor progression. When a tumor was palpated, the interval between tumor cell injection and tumor development was considered as the tumor latent period. Tumor size was measured with calipers and tumor volume was determined by applying the (0.5236 (W + L) (W x L))/2 formula, where W represents the width and L is the length (Janik et al, 1975). Animals were euthanized between 21-23 days by decapitation while under anesthesia with methoxyflurane vapor. Tumors were dissected and weighed. Tumors were cut into small pieces and were snap frozen in liquid nitrogen for PCR analysis. A portion of each tumor was fixed in 10% neutral formalin. Tissues for histologic studies were embedded in paraffin, cut at 6.0 μm thick, and stained with hematoxylin and eosin. Photomicrographs of representative sections of the tumor tissues were taken with a camera mounted on a microscope (Olympus Model BH-2) as a hard copy record.

Statistical analysis: All numerical data were expressed as mean ± standard error of the mean (SEM). All *in vitro* experiments were repeated at least 3 times. Data were analyzed using the analysis of variance test and Duncan's new multiple range test (Steele and Torrie, 1960; Bender et al, 1982). The Chi-square test was used to determine differences in tumor incidences in animals. A linear correlation was conducted to test the degree of association between the value of ^{3}H-thymidine incorporation and the level of TGF-β1 production for different clones. A correlation coefficient (r) was calculated based on the analysis. A p value of less than 0.05 was considered as statistically significant (Steele and Torrie, 1960; Bender et al, 1982).

RESULTS

Verification of TGF-β1 antisense transfection in MATLyLu cells. A TGF-β1 antisense vector was created by inserting the full-length rat TGF-β1 cDNA into the multiple cloning site of the pTarget vector in the reverse orientation. MATLyLu cells were transfected with this construct, selected with G418-sulphate, and re-transfected in order to assure that high copy numbers of the vector were present. The repeated transfection appeared necessary, as MATLyLu cells produce high levels of TGF-β1. Early attempts using a single transfection episode were unsuccessful in reducing TGF-β1 production in transfected cells. It is likely that a great measure of antisense RNA must be present in the cytoplasm in order to suppress TGF-β1 production. Following serial dilution, clones were chosen. DNA from these cloned cells was isolated for PCR analysis. The positive detection of the expected 399 bp PCR product derived from transfected cell lines confirmed the presence of the transfected construct. Levels of TGF-β1 in cell lysates, as determined by ELISA, were significantly lower in clones transfected with the TGF-β1 antisense vector than in those of wild type MATLyLu cells and cells transfected with the control construct. The clone with the lowest level of TGF-β1 (clone 18) was chosen for further studies (Table 1 and Fig. 1).

^{3}H-Thymidine Incorporation assay. ^{3}H-thymidine incorporation in these cells was measured to determine proliferation rates of the various positive clones. There was a negative correlation between values of ^{3}H-thymidine incorporation and TGF-β1 levels in different clones ($r=0.769$, $n=7$, $p < 0.05$). Clone 18 has the highest level of ^{3}H-thymidine incorporation, which was 3- to 5-fold higher than the proliferation rates for the wild type cells and cells transfected with the control construct (Table 1 and Fig. 1). Therefore, this clone was chosen for further investigation.

Tumor latent period, incidence, and progression of MATLyLu cells in animals: The tumor latent period is defined as the interval between tumor cell inoculation and detection of palpable tumor nodules. Table 2 shows that, in male Copenhagen rats (syngeneic hosts), 100% of tumor development

from wild-type MATLyLu cells (15/15) and cells transfected with the control construct (15/15); but only 43% in animals injected with clone 18 cells (9/21). The average latent period for tumor development was 12±0.54 days (mean±SEM) for the wild type MATLyLu cells and cells transfected with the control construct, for clone 18 cells, the average latent period was 25.2±3.4 days ($p < 0.05$). Furthermore, tumors derived from clone 18 cells had an average weight (2.5± 1.3 g at day 41) significantly less than those derived from wild-type MATLyLu cells (15.5±4.3 g) and cells transfected with the control construct (12.6±3.7 g) at day 21 (Fig. 2A).

The experiment was repeated in male nude rats (immunodeficient hosts) and in Lewis rats (allogeneic hosts). As indicated in Table 2, tumor incidence of clone 18 cells grown in nude rats was 90% (9/10), which was not significantly different from those of wild-type MATLyLu cells (10/10) or of cells transfected with the control construct (10/10). The latent period (8-11 days) of tumor emergence was not significantly different, in contrast to the extended latent period observed in the syngeneic hosts. Furthermore, the tumor mass of clone 18 cells was significantly smaller ($p < 0.05$) from that of wild type cells or cells transfected with the control construct (Fig. 2B). This observation suggests that for clone 18 cells, the host immune responses as well as non-immune responses contributed to the reduced tumor growth in Copenhagen rats. Tumor incidence in Lewis rats (allogeneic hosts) was also studied in a small series. Results indicated that, in Lewis rats, there was a 100% tumor development for wild-type MATLyLu cells (4/4), 80% for cells transfected with the control construct (4/5) and 0% in animals injected with clone 18 cells (0/5) (Table 2).

Characterization of MATLyLu tumors: Histologic features of tumors were similar to those reported in the literature (5,7). They contained highly undifferentiated tumor cells, resembling those of Gleason 5 anaplastic prostate adenocarcinoma. Mitotic figures were frequently seen. When tumor size was greater than 1.0 cm in diameter, central necrosis was evident. The periphery of the tumor was always surrounded by fibrous tissue derived from the host. In the present study, since tumors were harvested at a

relatively early stage, the incidence of metastasis was not frequent. There were no apparent differences in morphologic features of tumors harvested from different treatment groups.

Results of PCR analysis for the presence of the expression vector indicated that clone 18 cells contained the expected 399 bp PCR product. Levels of TGF-β1 in the tumor lysates were determined by ELISA. As shown in Figure 3, in syngeneic as well as in athymic rats, tumors derived from the three different cell types showed comparable levels of TGF-β1. These results suggest that tumors derived from these cells all contain relatively high levels of TGF-β1 regardless of whether or not the original inoculum produced a low level of the growth factor.

DISCUSSION

Results of the present study have demonstrated that MATLyLu cells, when the production of TGF-β1 is reduced, proliferate more rapidly under *in vitro* conditions but are less tumorigenic under *in vivo* conditions. The difference in growth behavior of tumor cells under these two conditions is likely due to tumor-host interactions. It is widely accepted that wild type MATLyLu cells are non-immunogenic or, at best, weakly immunogenic (Shaw et al, 1987; Vieweg et al, 1994). Many experimental immunotherapy protocols have failed to cure the MATLyLu tumor (Vieweg et al, 1994). Results of the present study have demonstrated that the wild type MATLyLu cells produce high levels of TGF-β1 and are immuno-suppressive (Steiner and Barrack, 1992; Barrack, 1997). By simply reducing the production of the endogenous TGF-β1, MATLyLu cells showed a reduced tumor incidence in immune competent hosts but not in immune deficient hosts.

TGF-β is a potent immunosuppressant (Karpus et al, 1991; Leterio and Roberts, 1998). The notion that many tumor cells produce large quantities of TGF-β has been acknowledged (Gomella et al, 1992). Therefore, it is reasonable to propose that many tumor cells are immunosuppressive and that they are able to escape the host immune surveillance system. Basically, the present study observed the effect of TGF-β1 production by the tumor cells on three tumorigenic events: the incidence of tumor formation, the latent period, and the progression of established tumors. The first two events are clearly T cell mediated, as there are significant differences in tumor incidence and the latent period between the syngeneic hosts and immune-deficient hosts. The third event may involve factors other than T-cell mediated immune system, as the impact of a reduced production of TGF-β1 on tumor progression is apparent in both syngeneic hosts and in immune deficient hosts. These issues will be addressed below.

Xenograft growth of tumor cells in athymic hosts offers an opportunity to assess the behavior of tumor development under immune compromised conditions. These animals are deficient in T cells but their natural killer (NK) cells remain functional. If tumors fail to develop in immune competent hosts but

grow in immunodeficient hosts, it is likely that the observed difference in tumor incidence is at least due to a functional T-cell immune system. In the present study, this is the case for tumor incidence and the latent period for tumor development, as the incidence has been significantly reduced and the latent period has been significant delayed in syngeneic hosts when compared with those events in the nude rats. Therefore, a reduction in TGF-β1 production in the tumor cells results in an escape from the T cell mediated immunosuppression.

It is clear that the tumor-host interaction involves factors other than the T-cell immune system. These non-T cell factors may also play an important role in tumorigenicity. Nude rats, although lacking T cells, retain some immune function, as they still have NK cells, which may perform some tumor suppressive functions. It is likely that the observed retardation in tumor growth in nude rats receiving clone 18 cells may result from the activation of NK cells due to the low levels of TGF-β1 produced by these cells. Differences in tumor growth may not completely attributed to the host immune system. It is apparent, from the present result, that the host immune system is one of many host factors that can be influenced by the production of TGF-β1 by tumor cells (Karpus et al, 1991; Leterio and Roberts, 1998).

In addition to immune suppressive function, TGF-β1 production by tumor cells can be responsible for many non-immune host factors, which can not be ruled out at this stage. These are angiogenesis, stromal-epithelial interaction, expression of adhesion molecules, and production of extracellular matrix proteins (Battegay et al, 1990; Yang and Moses, 1990; Welch et al, 1990; Karpus et al, 1991; Barrack, 1997). These factors can promote tumor progression. In an environment of reduced TGF-β1 production, the in vivo tumor growth will be hampered. Results from studies with immunodeficient hosts indicate that tumors derived from the wild type MATLyLu cells and cells transfected with the control construct grow faster than those derived from the TGF-β 1 antisense transfected cells do. These results indicate that the high levels of TGF-β1 produced by the wild type MATLyLu cells and by cells transfected with the

control construct are able to stimulate a greater degree of tumor growth than that of the TGF-β1 antisense transfected MATLyLu cells. These factors may also contribute toward MATLyLu tumor growth.

An interesting finding in this study is that MATLyLu cells, when their TGF-β1 production is reduced, exhibit a low tumor incidence and a prolonged tumor latent period in syngeneic hosts. When these tumor lysates were subjected to ELISA, however, the average level of TGF-β1 was not significantly different from that of the wild type tumors. Although results of PCR analysis indicate the presence of the antisense expression construct, the possibility could not be ruled out that some of the clone 18 cells might have lost their antisense constructs. We also acknowledge the possibility that a small fraction of the low TGF-β1 producing cells were never successfully transfected with the antisense vector. In either case, the consequence is the emergence of wild type cells in these tumors. The interesting aspect of this observation is that growth of these tumors appeared to be suppressed. A question has been raised that is whether or not these TGF-β1 producing clone 18 cells would grow tumors as large as those derived from wild type MATLyLu cells, if they are left in Copenhagen rats for longer periods. We speculate that they would not grow as aggressive as the wild type tumors. This is because when the low TGF-β1 producing clone 18 cells were injected into syngeneic hosts, they have been immunized, capable of rejecting wild type tumor cells at least for a few months. This line of rationale is based on the report by Fakhrai et al (1996). These authors used the same method to transfect TGF-β2 antisense vectors into rat glioma cells and observed a similar reduction in tumorigenicity in syngeneic hosts. Subsequently, they re-challenged the wild type glioma cells to hosts, who had previously rejected tumors. These re-challenged cells were also rejected, suggesting that, at that stage, host animals have been immunized to reject tumor cells even if they produce high levels of TGF-β.

In the present study, the acquisition of immunogenicity in low TGF-β1 producing MATLyLu cells was further substantiated by a lack of tumor growth in allogeneic hosts. In a preliminary study, tumors derived from wild type MATLyLu cells were eventually rejected by allogeneic hosts (Lewis rats) at 28

days following the initial tumor cell inoculation. The growth of MATLyLu tumors in Lewis rats is a typical allogeneic transplantation rejection, which is characterized by an acute T cell mediated reaction (Sherman and Chattopadhyay, 1993). However, during the first 14 days following inoculation of tumor cells, tumors grew from the wild type MATLyLu cells and cells transfected with the control construct, but not for TGF-β1 antisense transfected cells. This observation suggests that the high TGF-β1-producing tumors are endowed with a potent immunosuppressive shield, which protects them from the initial phase of allogeneic rejection.

In conclusion, the present observations have allowed us to conclude that MATLyLu cells, upon down-regulation of TGF-β1 production, become growth stimulated under *in vitro* conditions and growth inhibited under *in vivo* conditions. The *in vivo* growth inhibition is likely due to the presence of tumor-host interaction. The tumor-host interaction includes the host immune system and non-immune responses. Therefore, we propose that MATLyLu cells are actually immunogenic and that this immunogenic property has been masked by the endogenous production of a high level of TGF-β1. The high level of TGF-β1 in MATLyLu cells is a critical factor that renders these cells non-immunogenic. Reducing the endogenous production of TGF-β1 restores the immunogenicity in these cells. Our future studies will focus on cellular and humoral mechanisms that mediate such restoration of immunogenicity in MATLyLu cells. Understanding this mechanism may lead to the development of therapeutic programs based on lowering the level of TGF-β production in tumor cells.

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Table 1

Levels of TGF-β1 in cell lysates and ^{3}H-thymidine incorporation rates of wild type MATLyLu cells, cells transfected with the control construct and cells transfected with the TGF-β1 antisense construct.

Cell Type	TGF-β1 (pg/2x10^{4} cells)	^{3}H-Thymidine Incorporation (cpm/1x10^{4} cells/well)
Wild Type MATLyLu Cells	70.3 ± 7.7	6,804 ± 434
Cells with Control Vector	67.0 ± 11.8	10,774 ± 700
Cells with Antisense Vector (Clone 18)	9.9 ± 1.2*	30,348 ± 2,866*

All values are expressed as mean ± standard error of the mean.

*The value is significantly different from the other values in the same column ($p < 0.05$).

Table 2

Tumor incidence of MATLyLu cells inoculated subcutaneously into syngeneic, allogeneic, and immunodeficient hosts.

	Trial I	Trial II	Trial III	Overall
Syngeneic Hosts:				
Wild type MATLyLu cells	5/5(100%)	5/5(100%)	5/5(100%)	15/15(100%)
TGF-β1 antisense transfected cells	3/5(60%)	4/11(36%)*	2/5(40%)	9/21(43%)*
Control construct transfected cells	5/5(100%)	5/5(100%)	5/5(100%)	15/15(100%)
Allogeneic Hosts:				
Wild type MATLyLu cells	4/4(100%)			4/4(100%)
TGF-β1 antisense transfected cells	0/5(0%)*			0/5(0%)*
Control construct transfected cells	4/5(80%)			4/5(80%)
Immunodeficient Hosts:				
Wild type MATLyLu cells	5/5(100%)	5/5(100%)		10/10(100%)
TGF-β1 antisense transfected cells	5/5(100%)	4/5(100%)		9/10(90%)
Control construct transfected cells	5/5(100%)	5/5(100%)		10/10(100%)

All values are expressed as mean ± standard error of the mean.

*The value is significantly different ($p < 0.05$) from other values in the same group by the χ^2-square test.

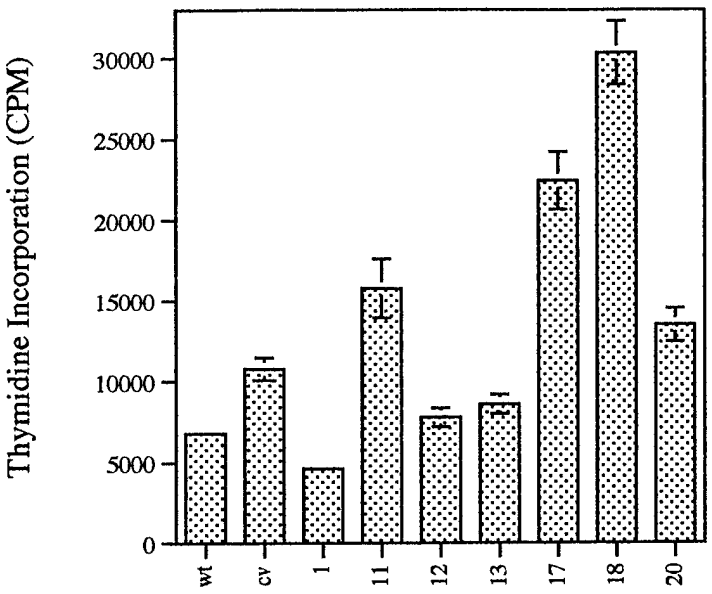
In syngeneic hosts and immunodeficient hosts, a total of $2x10^5$ cells were injected s.c. In allogeneic hosts, a total of $1x10^6$ cells were injected s.c. Three separate trials were conducted for syngeneic hosts. One trial was conducted for the allogeneic hosts and two trials were conducted for immunodeficient hosts.

Legend to Figures

Figure 1. The proliferation rates of wild type cells (WT), cells transfected with the control vector (CV), and different antisense clones *in vitro* (**A**, ^{3}H-thymidine incorporation) and levels of TGF-β1 production (**B**, ELISA). Clone 18 (c18) was selected because it was the fastest growing and had the lowest level of TGF-β1 production of all of the antisense clones that were screened.

Figure 2. Growth curves of tumors in Copenhagen rats (**A**, syngeneic hosts) and in nude rats (**B**, immunodeficient hosts). Tumor volumes were calculated according to the formula described in the text (18). Tumor volume in each group was calculated as mean ± SEM of all tumors. Tumors developed from the wild type MATLyLu cells (open squares) and cells transfected with the control vector (open triangles) were significantly bigger with a shorter latent period than those developed from the antisense vectors (open circles). Note that the scales in A and B are different, suggesting that tumors grow faster in immunodeficient hosts during the same interval. The vertical bars denote standard error of the mean.

Figure 3. Levels of TGF-β1 in tumors derived from Copenhagen rats (**A**, syngeneic hosts) and from nude rats (**B**, immunodeficient hosts). There were no statistical differences in TGF-β1. TGF-β1 levels (pg/50 mg tumor tissue) from wild type cells (WT), cells transfected with the control vector (CV), and cells transfected with the antisense vector (c18). Results are expressed as mean ± SEM for at least 5 tumors in each group. TGF-β1 was determine by ELISA as described in the text. The vertical bars denote standard error of the mean.


Thymidine Incorporation (CPM)
30000
25000
20000
15000
10000
5000
0
wt
cv
1
11
12
13
17
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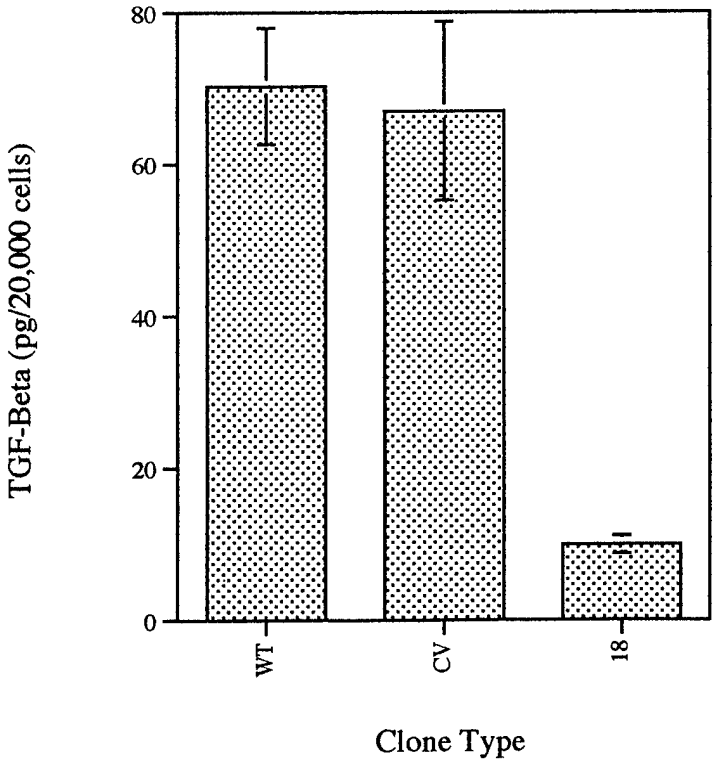
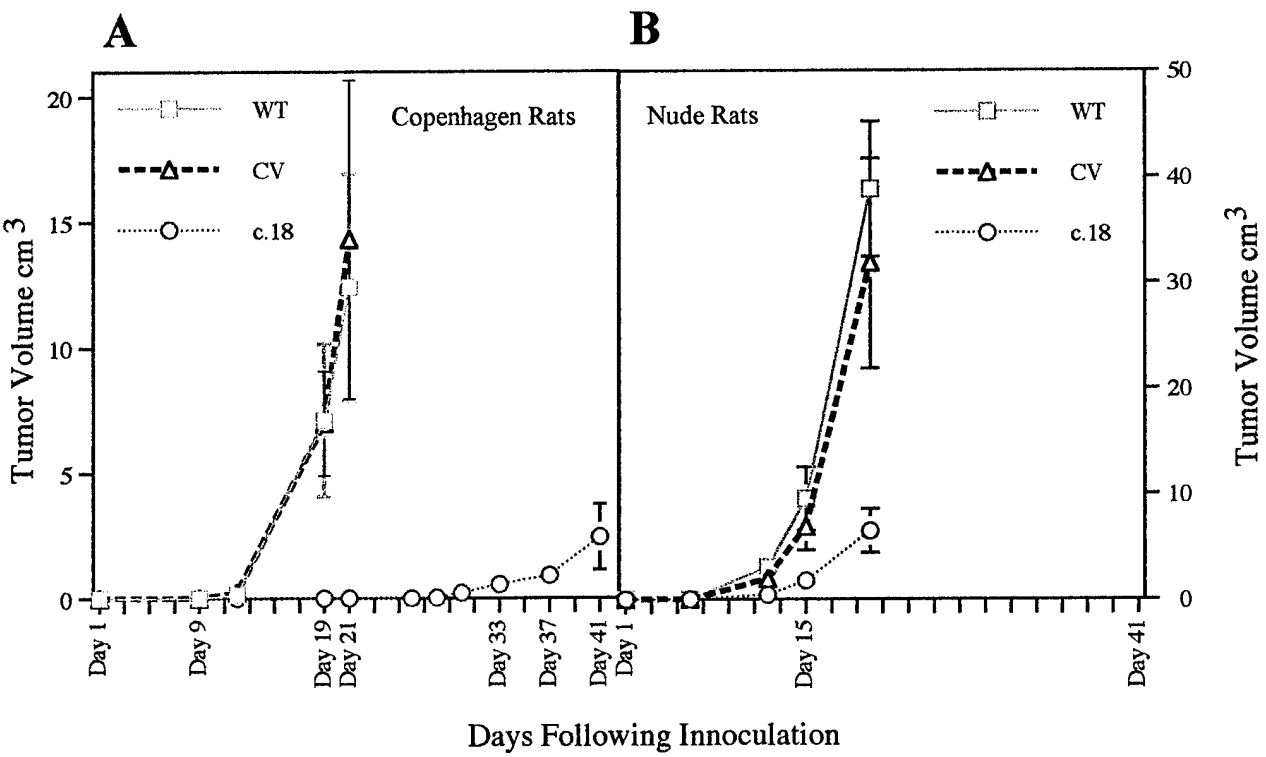
20
Clone Type

A


TGF-Beta (pg/20,000 cells)
80
60
40
20
0
WT
CV
18
Clone Type

B

Figure 1


A
B
WT
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Copenhagen Rats
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Tumor Volume cm 3
Tumor Volume cm3
0
5
10
15
20
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40
50
Day 1
Day 9
Day 19
Day 21
Day 33
Day 37
Day 41
Day 15
Days Following Innoculation

Figure 2

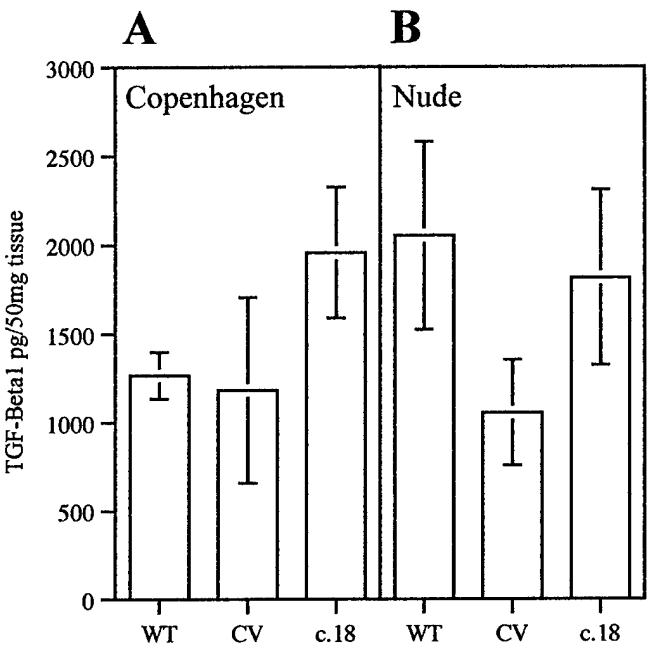
A
B
Copenhagen
Nude
TGF-Beta1 pg/50mg tissue
3000
2500
2000
1500
1000
500
0
WT
CV
c.18
WT
CV
c.18

Figure 3